Signaling through the Lymphotoxin β Receptor Induces th Death of Some Adenocarcinoma Tumor Lines

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Summary

Surface lymphotoxin (LT) is a heteromeric complex of LT-α and LT-β chains that binds to the LT-β receptor (LT-β-R), a member of the tumor necrosis factor (TNF) family of receptors. The biological function of this receptor-ligand system is poorly characterized. Since signaling through other members of this receptor family can induce cell death, e.g., the TNF and Fas receptors, it is important to determine if similar signaling events can be communicated via the LT-β-R. A soluble form of the surface complex was produced by coexpression of LT-α and a converted form of LT-β wherein the normally type II LT-β membrane protein was changed to a type I secreted form. Recombinant $LT-\alpha_1/\beta_2$ was cytotoxic to the human adenocarcinoma cell lines HT-29, WiDr, MDA-MB-468, and HT-3 when added with the synergizing agent interferon (IFN) γ. When immobilized on a plastic surface, anti–LT-β-R monoclonal antibodies (mAbs) induced the death of these cells, demonstrating direct signaling via the LT-β-R. Anti–LT-β-R mAbs were also identified that inhibited ligand-induced cell death, whereas others were found to potentiate the activity of the ligand when added in solution. The human WiDr adenocarcinoma line forms solid tumors in immunocompromised mice, and treatment with an anti–LT-β-R antibody combined with human IFN-γ arrested tumor growth. The delineation of a biological signaling event mediated by the LT-β-R opens a window for further studies on its immunological role, and furthermore, activation of the LT-β-R may have an application in tumor therapy.

The TNF family of ligands and receptors is a set of regulatory elements in the immune system (1). TNF was discovered as a cytolytic agent circulating in the blood of endotoxin-stimulated animals (2–4). Originally cloned in the expectation that TNF would be a novel antitumor agent, it was later shown that its primary physiologic function lies in initiating the inflammatory cascade underlying the host's immediate defensive response to infection or stress. More complex immunological functions have been described (5, 6). Lymphotoxin (LT)[1] α (also called TNF-β) is a similar cytokine secreted by activated lymphocytes (7) and was originally characterized as having the same functions as TNF. Later, activated T and B cells were found to display LT-α on their surfaces in an unusual form complexed with another member of the TNF family called LT-β in an $LT-\alpha_1/\beta_2$ stoichiometry (8–13). A complex with an apparent $LT-\alpha_2/\beta_1$ stoichiometry is also present, but only in minor amounts on human lymphocytes. The major $LT-\alpha_1/\beta_2$ form does not bind to the known TNF receptors, referred to here as TNF-R55 and TNF-R75, but rather interacts with another receptor in the TNF family called the LT-β receptor (LT-β-R) (9, 14).

Currently, the function of the LT system is poorly characterized, however, there are suggestions that LT signaling is involved in the development of the peripheral lymphoid organs. Genetic disruption of the LT-α gene in mice led to an unusual phenotype. The mice lacked lymph nodes and lost the organization of T and B cells in the follicles in the spleen (15, 16). A similar loss of lymph nodes occurs in *aly*, mice although this mouse, unlike the LT-α knockout mouse, is severely immunocompromised (17). Signaling through the two known TNF receptors has not been shown to mediate the development of the lymph nodes since knockout of either receptor does not lead to the loss of lymph nodes (18, 19). Thus, it has been postulated that signaling through the LT-β-R constitutes a regulatory pathway that is distinct from TNF-related events and may

account for the unique phenotype of the LT-α knockout mouse (12, 15, 16).

Activation of several members of the TNF family of receptors can have cytotoxic or growth-inhibitory consequences (1). For example Fas receptor activation results in apoptosis of many cell types, including both transformed and nontransformed cells (20, 21), and this process is likely to play a role in the deletion of autoreactive lymphocytes in the periphery (22). TNF and LT-α also can kill some transformed cells, and it is likely that tumor cells respond abnormally by either necrosing or apoptosing to what is normally a differentiation-like signal. More recently, TNF signaling has been proposed to induce the death of nontransformed lymphoblasts in a slow fashion (23, 24), and this process appears to require TNF-R75. The physiological significance of this event remains to be explored. The Fas receptor and the TNF-R55 both possess a unique cytoplasmic domain, called the death domain, that is required to initiate cell death (25). CD30 and CD40 signaling can inhibit growth and may also induce apoptosis, yet these receptors as well as the TNF-R75 and the LT-β-R lack obvious death domains (12, 26, 27). We have investigated whether LT-β-R signaling could induce cell death both because of its possible immunological relevance and to provide a practical starting point for studying the role of the LT system. Using either recombinant ligands or antireceptor mAbs with agonist activity, the ability of LT-β-R activation to induce cell death in various transformed lines was examined. In this report, we show that LT-β-R signaling can induce cell death in a limited group of adenocarcinoma tumor lines.

Materials and Methods

Cells. All cells were obtained from American Type Culture Collection (ATCC) (Rockville, MD) except for WEHI 164 clone 13, which was obtained from Dr. Eric Kawashima (Glaxo Institute for Molecular Biology, Geneva, Switzerland). WEHI 164 cells were cultured in RPMI 1640 with 10% fetal bovine serum (FBS). HT-29 and WiDr cells were maintained in MEM with Earle's salts, 10% FCS with glutamine, penicillin/streptomycin, nonessential amino acids, and sodium pyruvate. These two cell lines are thought to be derived from the same patient (28). In our assays, the original ATCC HT-29 line was heterogenous in its response to LT-α₁/β₂. and not all of the cells died in a parallel manner. Subclones from the line were isolated by limiting dilution, and the HT-29-14 line was one subclone that behaved homogenously in these assays. All of the results can be reproduced qualitatively with the parental line.

Materials. The anti-Fas mAb CH11 was obtained from Kamiya Biomedical Co. (Thousand Oaks, CA), the control IgG1 mouse mAb MOPC 21 from Organon Technica (Durham, NC) and the anti-CD40 mAb BB20 from R&D Systems (Minneapolis, MN). The anti-human LFA-3 mAb 1E6 has been described (29) and the anti-LFA-3 mAb TS2/9 was provided by Barbara Wallner. The anti-TNF mAb 104c has been described (30). The HT-29/26 hybridoma that produces a mAb that recognizes an abundant antigen on the HT-29 surface was obtained from ATCC, cells were grown, and the mAb purified by protein A–Sepharose chromatography. The LT-β-R–hIgG1 and TNF-R55–hIgG1 Fc fusion proteins have been described (9).

Recombinant Cytokines. Human TNF and IFN-γ were produced at Biogen (30). Recombinant human LT-α was prepared by expression in insect cells as described (31) and was similar to material expressed in CHO cells (30). The recombinant LT-α/β heteromeric forms were prepared by coinfection of insect cells with two baculoviruses encoding the human LT-α and human LT-β proteins (Browning, J.L.; K. Miatkowski, D.A. Griffiths, P.R. Bourdon, C. Hession, C.M. Ambrose, and W. Meier, manuscript in preparation). The transmembrane region of the LT-β gene was replaced with a vascular cell adhesion molecule (VCAM) leader sequence to enable secretion of mixed LT-α/β forms. The trimers LT-α₁/β₂, LT-α₂/β₁, and LT-α₃ were purified using combinations of p55 TNF-R and LT-β-R affinity columns. The resultant preparations have been well characterized, contain only LT forms, are trimeric, and are >95% pure with respect to LT forms based on ion exchange chromatographic resolution of the three stoichiometrically different trimers. The LT-α₁/β₂ preparation contained <1 part in 5,000 of LT-α₃-like activity as assessed using the WEHI 164 indicator line and by comparison of various LT trimers prepared with a LT-α D50N mutation that eliminates TNF-R binding (Browning J.L., K. Miatkowski, D.A. Griffiths, P.R. Bourdon, C. Hession, C.M. Ambrose, and W. Meier, manuscript in preparation).

Cytotoxicity Assays. In the cytotoxicity assays, serial dilutions of the cytokines or antibodies were prepared in 50 μl in 96-well plates, and 5,000 HT-29-14 cells were added in 50 μl of media with or without IFN-γ. After 3–4 d, 10 μl of 5 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl) 2,5 diphenyltetrazolium bromide] was added, and after 3 h the formazan was dissolved by adding 100 μl of 10% SDS in 10 mM HCl. After a further 24-h incubation at 37°C, the OD was quantitated at 550 nm. In some experiments, soluble receptor forms or pure human IgG were added in 10 μl before the addition of the cells. To immobilize mAbs on the plastic surface, 96-well tissue culture plates were first coated with 50 μl of 10 μg/ml affinity-purified goat anti–mouse Fc polyclonal antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), washed with 5% FBS in PBS, and then coated with varying amounts of the various mAbs diluted into tissue culture media with FBS. The plates were washed with media before use. Cells were added and growth assessed as indicated above. To survey the panel of cells shown in Table 4, cells were plated in the presence of 50 U/ml IFN-γ with various dilutions of TNF, anti-Fas, LT-α₁/β₂, or into wells coated with various anti–LT-β-R mAbs as described above. Growth was assessed with the MTT readout, and wells were also visually inspected for morphology changes. Dramatic growth inhibition was scored as two pluses (++), partial growth inhibition at reasonable concentrations was noted as one plus (+), and partial effects requiring high concentrations of ligand was marked as plus/minus (+/−).

Mouse Anti–LT-β-R mAbs. Mouse hybridomas producing mAbs to the human LT-β-R were prepared by immunization of RBF mice with the LT-β-R-Fc chimera essentially as described previously (9). All mAbs were IgG1 isotypes.

FACS® Binding Assays. To monitor receptor binding to surface ligand, 200 ng/ml soluble LT-β-R–Fc were added to PMA-activated II-23 T cell hybridoma cells, and binding was detected using a PE-labeled donkey anti–human Ig essentially as described (9). To assess the blocking ability of the mAbs, mAbs were preincubated with the soluble receptor for 20 min before addition of the receptor–mAb mixture to the II-23 cells. In other experiments, receptor expression on adherent tumor lines was determined by FACS® analysis of cells removed with PBS with 5 mM EDTA and stained using anti–LT-β-R mAbs and a PE-labeled

donkey anti—mouse IgG reagent (Jackson ImmunoResearch Laboratories).

Epitope Mapping by BIAcore™ Analysis. Affinity-purified goat anti–human Fc (Jackson ImmunoResearch Laboratories) was immobilized onto an N-hydroxysuccinimide–activated sensor chip, and LT-β-R-Fc was captured onto the anti–human-Fc–coated chip. The various pairs of anti–LT-β-R mAbs were then bound sequentially, and the ability of the second mAb to bind in the presence of the first mAb was measured using a BIAcore™ 2000 (Pharmacia Biosensor, Uppsala, Sweden). The entire array of 49 mAb combinations were assessed for cross-blocking in this manner and analyzed essentially as described (32).

Analysis of Apoptosis. Terminal deoxynucleotidyl transferase UTP nick-end labeling (TUNEL) of free DNA ends, i.e., TUNEL staining, was carried out using the ApopTag™ kit (Oncor Inc., Gaithersburg, MD) according to the manufacturer's specifications.

Tumor Growth in SCID Mice. BALB/c SCID female mice at 6–8 wk old (The Jackson Laboratory, Bar Harbor, ME) were injected with 10^6 trypsinized and washed WiDr cells in a volume of 0.2 ml of PBS subcutaneously onto the back of the animal. Mice were treated with or without antibody either with or without 10^6 antiviral U/mouse of human IFN-γ by intraperitoneal injection in 0.2 ml on days 0 and 1 or as indicated. The amounts of IFN-γ and antibody have not been optimized. Tumor volume was calculated from the radius as determined by caliper measurements in two dimensions. The results shown in Fig. 6 B were determined in a blinded format.

Results

LT-α₁/β₂ Is Cytotoxic to HT-29 Cells. Recombinant LT-α₁/β₂ and LT-α₂/β₁ trimers were tested for their ability to inhibit the growth of a number of tumor lines. IFN-γ was included in the screening as it had been shown to enhance the cytolytic properties of TNF (33). Recombinant soluble LT-α₁/β₂ inhibited the growth of human HT-29 cells only in the presence of IFN-γ (Fig. 1) and, as shown previously, this cell line was also sensitive to the anti-Fas receptor mAb CH11, TNF, and LT-α (30, 34, 35). IFN-α and -β were 100-fold less effective when compared on the basis of antiviral units (data not shown). Soluble LT-α₂/β₁ was much less active in this assay.

The specificity of the LT-α₁/β₂ cytotoxicity was examined in several ways. Soluble TNF-R55 and LT-β-R immunoglobulin chimeras (TNF-R55-Fc and LT-β-R-Fc)

were tested for their ability to block the various activities (9). These receptors can bind to the appropriate cleft between two subunits in the trimeric ligand structures and interfere with the ability of receptor on the membrane to bind ligand. As expected and shown previously (36, 37), TNF-R55-Fc completely blocked TNF-induced growth inhibition by binding to TNF and preventing its interaction with surface receptor (Table 1). Soluble TNF-R had no effect on LT-α₁/β₂-mediated antiproliferative effects. In contrast, LT-β-R-Fc blocked LT-α₁/β₂ effects, but not those of TNF or LT-α. Moreover, anti–LT-α–neutralizing mAbs (9) did not affect the LT-α₁/β₂ cytotoxicity (data not shown), confirming that soluble trace LT-α contaminants were not involved in the activity of LT-α₁/β₂ on HT-29 cells. Additionally, a mutated form of LT-α (D50N) that lacks the ability to signal through the TNF-R was examined (38). LT-α₁/β₂ prepared with the mutant LT-α retained essentially full activity on HT-29 and WiDr cells (see below), further eliminating TNF-R55 binding as a possible mechanism for the cytotoxic effects of LT-α₁/β₂ (Ambrose, C., unpublished data). An anti-TNF neutralizing mAb 104c, also had no effect on LT-α₁/β₂ activity, precluding the induction of TNF synthesis as a mechanism for the LT-α₁/β₂ effects. These assays indicate that LT-α₁/β₂ can trigger cytotoxic events via non–TNF-R–mediated mechanisms.

Mechanism of Growth Inhibition. The growth inhibition assay alone does not discriminate between death and stasis; however, direct observation of the treated HT-29 cells showed that Fas receptor activation led to rapid cell death, i.e., within 12–24 h, whereas TNF effects were slower and required ~24 h. LT-α₁/β₂-treated cells underwent a much slower death, with dead cells not being visible until 1.5–2 d. The morphology of the dying cells was identical in all three cases. Some cells have the appearance of apoptotic bodies with condensed nuclei, whereas in others the nucleus appears to condense and the cytoplasm balloons out and becomes clear (Fig. 2). Substantial cell lysis does not occur even after 3–4 d. The TNF-, anti-Fas receptor mAb– or LT-α₁/β₂-treated populations had more cells that stained brightly with HOECHST dye staining (data not shown), which can be indicative of chromatin condensation accompanying apoptotic events. Internucleosomal DNA fragmen-
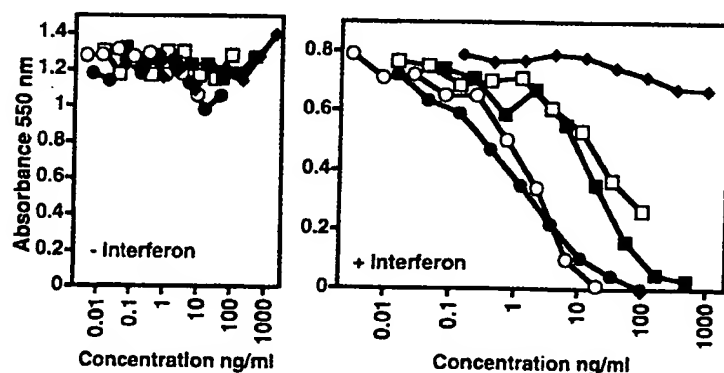

Figure 1. The cytotoxic effects of LT-α₁/β₂ on the human adenocarcinoma HT-29 cells. Comparison of the cytotoxic activity of anti-Fas receptor mAb CH-11 ( ), TNF (○), LT-α (□), LT-α₁/β₂ (■), and LT-α₂/β₁ (◆) in the presence and absence of 80 U/ml IFN-γ.

Table 1. *Ability of LT-β-R and TNF-R55 Immunoglobulin Fusion Proteins to Block the Inhibitory Effects of Various TNF and LT Ligands on HT-29 Growth*

	Concentration of cytotoxic agent resulting in 50% growth inhibition		
	Presence of*		
Cytotoxic agent	hu-IgG control	TNF-R55–Fc	LT-β-R–Fc
	ng/ml	*ng/ml*	*ng/ml*
TNF	0.08	>10‡	0.08
LT-α	3.0	>1000	3.0
LT-α₁/β₂	5.0	5.0	>200.0
Anti-Fas mAb	2.0	2.0	2.0

*Each cytotoxic agent was premixed with the Ig fusion proteins for 10 min before addition to the cells. The final concentration of fusion protein was 5 μg/ml.

‡Higher concentrations were not tested.

tation or laddering was not observed after LT-α₁/β₂, TNF, or anti-Fas treatment of HT-29 or WiDr cells, although a prominent large DNA band was visible (data not shown) after all three treatments. This large fragment is reminiscent of the large 50–200-kb cleavage products previously described in dying epithelial tumor cells (39, 40). Some DNA fragmentation was observed using TUNEL staining of 3′ hydroxyl ends of DNA, which is more sensitive than DNA laddering (Fig. 3). Therefore, the death induced by TNF-R, Fas, or LT-β-R signaling may be basically apoptotic even though all of the classic features of apoptosis have not been observed in epithelial tumors (39, 41).

Properties of Anti–LT-β-R mAbs. Antibodies to receptors in the TNF family can have either antagonistic or agonistic effects, and as tools they have been very useful in delineating the consequences of receptor activation. To determine if LT-β-R cross-linking could induce cell death, we prepared and characterized similar LT-β-R–specific mAbs. Mice were immunized with the LT-β-R–Fc fusion protein, and a panel of mouse anti–LT-β-R mAbs were isolated. These mAbs were grouped into four subsets based on their performance in the following assays: (*a*) the ability to cross-block each other in a mAb/antigen–binding experiment using plasmon resonance detection (i.e., a BIAcore™ epitope mapping experiment); (*b*) the ability to block soluble LT-β-R–Fc binding to surface ligand on PMA-activated II-23 cells; and (*c*) the ability to affect LT-α₁/β₂-induced cell death. The results of this analysis are summarized in Table 2.

Epitope mapping using the BIAcore™ instrument defined four separate epitopes recognized by this panel of mAbs and formed the basis of the grouping shown in Table 2. Within each group, the mAbs effectively blocked each other. The group I mAbs did not cross-block any other mAbs. The group II epitope partially overlapped the group III epitope. We identified only one mAb, CBE11, with group IV properties, and its epitope slightly overlapped the

BCG6 and BKA11 sites. To directly assess the ability of the mAbs to inhibit receptor–ligand binding, a FACS® binding assay was used. Antibodies were premixed with soluble receptor, and the ability of the receptor to bind to ligand on the surface of II-23 cells was quantitated. This assay has the advantage that any agonistic activity of the antireceptor mAbs would not be apparent. mAbs from groups I, II, and IV were effective inhibitors, suggesting that they bind close to the ligand binding region of the receptor (Table 2).

In the HT-29 cytotoxicity assay, all mAbs in these groups either weakly inhibited growth or lacked activity when simply added to the culture in the absence of ligand (Fig. 4 *A*). There was considerable variability in this assay possibly reflecting differing receptor densities at the start of the experiment; e.g., compare Fig. 4 *A* and Table 3. In dramatic contrast, when the mAbs were immobilized on the plastic, all mAbs were found to have potent cytotoxic activity (Fig. 4 *B*), and this activity was again completely dependent on the presence of IFN-γ. This result demonstrates that LT-β-R cross-linking is sufficient to induce cell death. Moreover, we reasoned that mAbs to two different epitopes should be able to cross-link receptors more effectively, and indeed Table 3 shows that certain pairs of mAbs were cytotoxic in solution. In general, mAbs from two different groups had to be paired to get cytotoxic activity.

In addition to the direct agonistic effects of the mAbs described above, we investigated the effects of the mAbs on ligand induced cell death. Each of the four groups of mAbs had differing effects reinforcing the grouping based on BIAcore™ epitope mapping. Group I mAbs primarily blocked LT-α₁/β₂ activity with a small amount of direct growth inhibition occurring in the absence of ligand (Fig. 4 *C*). In some experiments (data not shown), a Fab fragment of BDA8 was used, and the small growth inhibitory activity disappeared and only direct inhibition was observed. Such complex mixed agonist/antagonist effects have been observed with anti-CD40 mAbs (42). The group II mAbs had complex effects that suggested mixed agonistic and antagonistic behavior (data not shown). Group III mAbs potentiated LT-α₁/β₂ activity presumably by creating local regions of high receptor density that would enhance ligand mediated cross-linking (Fig. 4 *D*), and there was no evidence of antagonistic effects. The cytotoxic effects of TNF were also slightly potentiated by these mAbs, suggesting that certain signal transduction elements were facilitating or priming the cells for TNF signaling. The group IV mAb, CBE11, did not affect ligand-mediated cytotoxicity. Because the LT-β-R–specific mAbs can directly effect death, one can conclude that the LT-β-R is able to signal cell death, and this event accounts for the cytotoxic activity of LT-α₁/β₂.

Survey of Sensitive Cell Lines. A series of human tumor lines have been screened for sensitivity to cytotoxic or growth-inhibitory effects of either the LT-α₁/β₂ ligand or plastic immobilized anti–LT-β-R mAbs in the presence of IFN-γ (summarized in Table 4). The WiDr line (43) was found to be sensitive to LT-α₁/β₂ in a very similar manner as the HT-29 line, and it is likely that this line is actually a
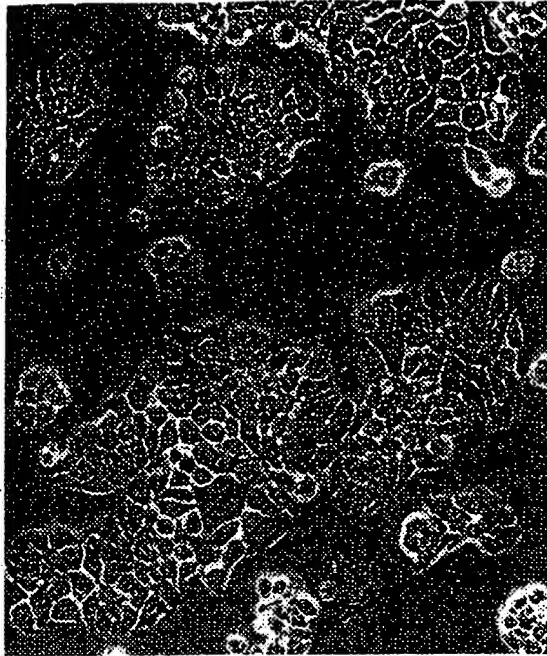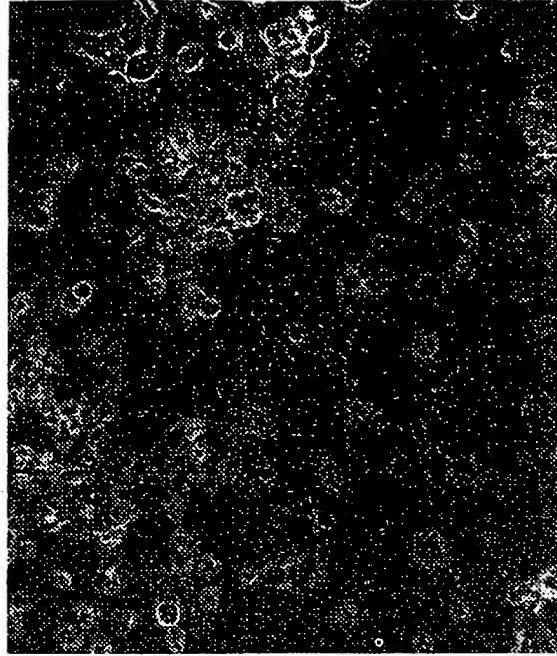
INTERFERON-γ   INTERFERON-γ + LTα1/β2

Figure 2. Photograph of HT-29 cells treated with IFN-γ (80 U/ml) alone or IFN-γ plus 50 ng/ml LT-α_1/β_2 for 3 d ×200.

derivative of HT-29 (28). The breast adenocarcinoma MDA-MB-468 and cervical carcinoma HT-3 lines were growth inhibited, and whether cell death occurred was not rigorously determined. In general, however, the majority of tumor lines are not sensitive under these relatively fixed conditions. This survey was complicated by the fact that the sensitivity of the cells to IFN-γ varied dramatically, and the lines are often heterogenous, with mixtures of responsive and nonresponsive cells obfuscating a simple MTT-based test.

Cell surface levels of LT-β-R were examined to determine whether LT-β-R presence is the determining factor for sensitivity to the LT ligand. Fig. 5 shows a comparison of LT-β-R and CD40 receptor levels on four different tumor lines. The CD40 staining is included since its surface levels are very abundant on some epithelial tumor lines, as is the LT-β-R. All four cell lines are LT-β-R positive, and these results are typical of most nonlymphoid cells (Mackay, F., and J.L. Browning, unpublished results). Despite the presence of LT-β-R on all four cell types, only HT-29 cells are appreciably sensitive to LT-α_1/β_2, indicating that sensitivity does not correlate simply with receptor presence. In the TNF, Fas, and CD40 signaling systems, no correlation has ever been observed between the level of surface receptor and whether a cell type responds biologically. Relative to Fas, TNF-R55, and TNF-R75, LT-β-R is very abundant on cells. Experiments on the HT-29 and WiDr lines indicated that IFN-γ treatment did not upregulate the LT-β-R, whereas in the same experiments the Fas receptor was dramatically upregulated as described previously (35).

There was no evidence that LT-β-R signaling was cytostatic to normal human fibroblasts, endothelial cells, or primary lymphocytes, which was expected since lymphocytes do not express the receptor (Hochman, P., J.L. Browning, and C. Ware, unpublished observations). The growth of the human diploid fibroblastoid line WI-38 was stimulated by LT-β-R signaling (data not shown). The adenocarcinoma lines SW620 and SW1417 both displayed altered morphology in response to LT-β-R signaling without growth arrest.

Anti-LT-β-R mAb Inhibits the Growth of WiDr in SCID Mice. We have explored the ability of an anti-LT-β-R mAb, CBE11, to block the growth of the WiDr line in immunodeficient mice. When the mice were treated intraperitoneally with the CBE11 mAb at the same time as the WiDr cells were inoculated subcutaneously, tumor outgrowth was dramatically blocked (Fig. 6 *A*). The antitumor action was enhanced by IFN-γ; however, the mAb was effective even without exogenous IFN-γ. In the CBE11 + IFN-γ group, 7 of 16 animals completely lacked tumors, whereas the remaining animals had small nodules that had not progressed at 2 mo. The CBE11 animals treated without IFN-γ were similar to the CBE11 + IFN-γ group at 30 d; however, these mice eventually developed slowly growing tumors. There were statistically significant differences between the control or IFN-γ-treated groups and the CBE11-treated groups, whereas no significant differences were observed between the saline, IFN-γ, or the control anti-human LFA3 mAb (1E6) + IFN-γ groups. The 1E6 and CBE11 mAbs are both IgG1s, and since the 1E6 mAb effectively coats the tumor line yet did not block tumor
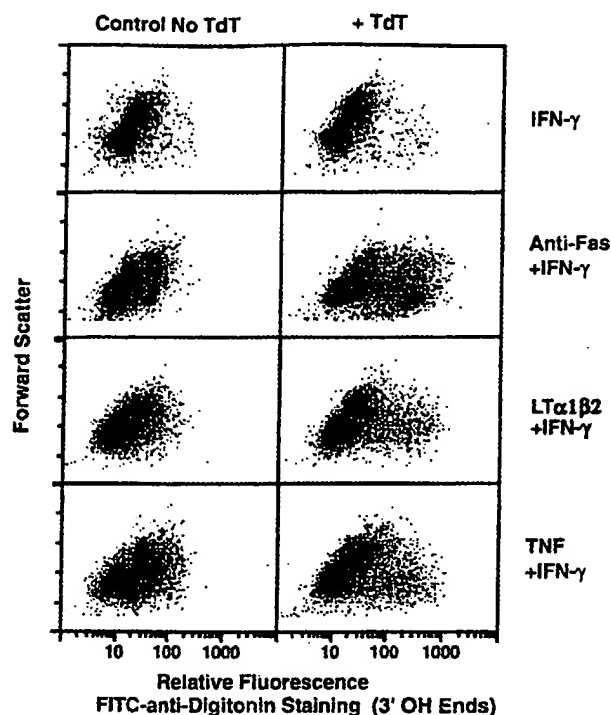
Control No TdT	+ TdT	
		IFN-γ
		Anti-Fas +IFN-γ
		LTα1β2 +IFN-γ
		TNF +IFN-γ

Forward Scatter (y-axis)

Relative Fluorescence
FITC-anti-Digitonin Staining (3' OH Ends) (x-axis)

Figure 3. FACS® analysis of TUNEL staining of DNA fragmentation occurring in response to Fas, TNF, or LT-α₁/β₂ signaling. HT-29 cells were exposed to 80 U/ml of IFN-γ for 3 d, 50 ng/ml anti-Fas mAb and IFN-γ for 2 d, 50 ng/ml LT-α₁/β₂ and IFN-γ for 3 d, or 10 ng/ml TNF with IFN-γ for 2 d. All cells in the culture were stained using the Apo-Tag™ kit (Oncor, Inc.) without or with terminal deoxynucleotidyl transferase addition.

growth, we can exclude complement- or NK cell–mediated events as the basis for the tumor inhibition. The efficacy of CBE11 in the absence of IFN-γ was unexpected since there was an absolute dependence on IFN-γ for any LT-β-R based in vitro cytotoxic effect. Either there is some crossover of mouse IFN-γ onto human IFN-γ receptors, or other mechanisms are involved in vivo. The mechanism(s) by which LT-β-R signaling prevents tumor growth in vivo effects are being investigated. The ability of combined IFN-γ/CBE11 treatment to inhibit the growth of an established tumor was demonstrated (Fig. 6 B). Mice were inoculated with 10⁶ WiDr cells, and after 15 d, treatment was initiated. At this point the average tumor volume was 0.076 cc or an average diameter of 0.532 cm. Treated tumors stopped growing, and after three injections of antibody over 3 wk, growth was arrested out to 7 wk after inoculation, at which point the experiment was terminated.

Discussion

Activation of the receptors of the TNF family can direct cells into a proliferative or differentiation type response, or it can induce cell death sometimes even in the same cell type depending on the conditions (4, 22, 44). In this work, we have shown that signaling through the LT-β-R leads to the death of the HT-29 and WiDr human adenocarcinoma cell lines and is at least growth inhibitory to two other lines. This activity represents the first observed effect of LT-β-R signaling and is important not only because of the current interest in cytotoxic events, but because it provides a biological assay for characterizing various reagents. There was no evidence that LT-β-R signaling was cytostatic to

Table 2. *Summary of Mouse Anti-Human LT-β-R mAbs*

				HT-29 cytotoxicity		
mAb group	mAb name	Cell staining*	Blocking receptor binding‡	mAb immobilized on Plastic§	Soluble mAb alone	Soluble mAb with LT-α₁β₂
I	BDA8	+++	+++	+	+/−¶	Inhibits
I	AGH1	+++	+++	+	+/−	Inhibits
II	BCG6	+++	++	+	+/−	Mixed
II	BHA10	+++	+++	+	+/−	Mixed
III	BKA11	+++	+/−	+	−	Potentiates
III	CDH10	+++	+/−	+	+/−	Potentiates
IV	CBE11	+++	+++	+	+/−	No effect
Controls						
	MOPC21	−	−	−	−	No effect
	HT29/26	−	ND	−	−	No effect
	TS 2/9‖	ND	ND	−	−	No effect

*FACS® staining of CHO cells transfected with LT-β-R.
‡Assay assessed whether antibody blocks binding of soluble receptor to the activated T cell hybridoma II-23.
§HT-29 cells were grown with IFN-γ on anti-LT-β-R–coated plates as described in Materials and Methods.
¶Variable, partial inhibition in some assays, no effects in others.
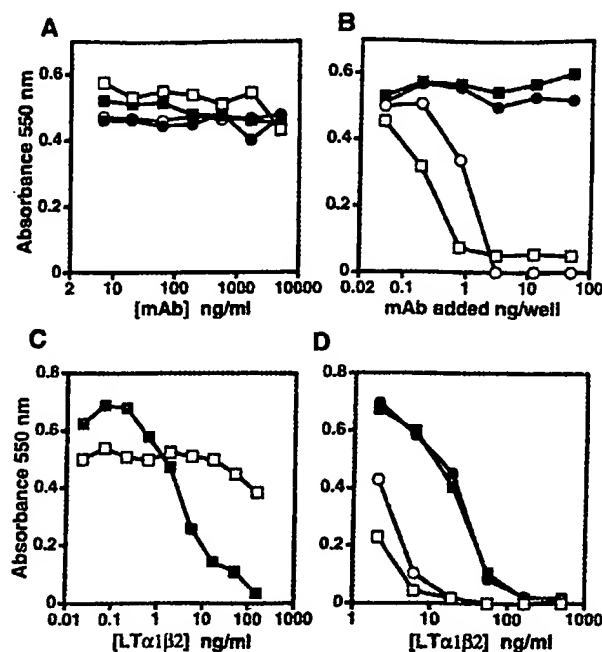‖Anti-human LFA-3, a mouse IgG1.

Figure 4. Effects of various anti-LT-β-R mAbs on HT-29 growth. *A* shows the effects of soluble anti-LT-β-R mAbs alone on the growth of HT-29 cells: control IgG1 (●), HT-29/26, a mAb to an irrelevant abundant surface Ag on HT-29 cells (■), BDA8 (O), and CDH10 (□). *B* illustrates the direct cytotoxic effects of these two anti-LT-β-R mAbs on HT-29 cells when immobilized on the plastic surface. Plates were coated with IgG1 (●), HT-29/26 (■), BDA8 (O), and CDH10 (□). *C* shows the LT-α_1/β_2 antiproliferative effects on HT-29 cells in the presence of 2 μg/ml control IgG1 (■) or anti-LT-β-R mAb BDA8 (□). BDA8 exhibits some agonist activity even at low concentrations. *D* shows the effects of CDH10 as an example of a group III anti-LT-β-R mAb that potentiates the effects of LT-α_1/β_2. LT-α_1/β_2 effects were measured in the presence of no mAb (●), 0.5 μg/ml control IgG1 (■), 0.05 μg/ml CDH10 (O), and 0.5 μg/ml (□) CDH10.

Table 3. *Effect of Pairs of Anti-LT-β-R mAbs on HT-29/WiDr Growth*

mAb	Concentration	mAb	Concentration	Cell growth
	(ng/ml)		(ng/ml)	(OD 550 nm)
IgG1	200			0.77 (HT-29)
BDA8	100	—	—	0.33*
—	—	BCG6	100	0.50
BDA8	100	BCG6	100	0.05
IGG1	50	—	—	0.85 (HT-29)
CDH10	33	—	—	0.525
—	—	AGH1	50	0.49
CDH10	33	AGH1	50	0.23
IgG1	100	—	—	0.80 (HT29)
CDH10	10	—	—	0.86
IgG1	50	CDH10	10	0.81
—	—	CBE11	50	0.58
CDH10	10	CBE11	50	0.21
BHA10	10	—	—	0.85
BHA10	10	CBE11	50	0.09
IgG1	50	—	—	0.62 (WiDr)
CDH10	33	—	—	0.36
—	—	AGH1	50	0.36
CDH10	33	AGH1	50	0.16

Dotted lines indicate separate experiments.
*Typically, further increases in the mAb concentration did not increase the amount of growth inhibition.

nontransformed cells, and signaling could actually drive growth in some situations. This pattern of cell sensitivity to LT-α_1/β_2 basically resembles the effects of TNF. The data we have obtained suggest that the cytotoxicity mediated by the LT-β-R will be limited to transformed cells. Recently, signaling through TNF-R75 has been reported to mediate an unusually slow death of nontransformed activated lymphocytes (23, 24), indicating that TNF-induced death may not be limited to transformed cells. In contrast, nontransformed lymphocytes can be induced to undergo rapid apoptosis after Fas signaling, and this event is most likely to be involved in the deletion of certain lymphocyte populations in the periphery (22, 45). Whether the slow events involving LT-β-R or TNF-R75 are physiologically important will require further investigation. Surface LT is abundant on lymphokine activated T cells, i.e., LAK cells (12, 46). Currently, cell mediated cytotoxicity is thought to be mediated through the perforin and/or Fas pathways (47). A classic LT-β-R-positive NK/LAK target, K562, was found to be completely resistant to the action of soluble LT-α_1/β_2, suggesting that neither surface LT is not involved in NK-like cytotoxicity. Genetic disruption of LT-α and the two

TNF-R does not impair CTL-mediated cell death supporting the hypothesis that TNF/LT signaling is not involved in cell–cell killing (13). Further experimental work will be required to determine whether LT signaling via direct cell–cell contact supports these conclusions.

The LT system with its heterotrimeric ligand is unusual. The cytotoxic activity resides primarily in the LT-α_1/β_2 form, with the LT-α_2/β_1 complex being much less active. The crystal structure of LT-α complexed with the TNF-R55 revealed that the receptor lies in the groove between two adjacent subunits (48). The higher potency of the LT-α_1/β_2 form suggests that the β/β cleft, which is unique in the LT-α_1/β_2 heterotrimer, must interact with the LT-β-R. Biochemical analyses of this interaction have confirmed that there is a high affinity interaction of LT-β-R with LT-α_1/β_2 and a lower affinity interaction with LT-α_2/β_1 (Browning, J.L., M. Zafari, C. Benjamin, W. Meier, D. Griffiths, and K. Miatkowski, unpublished observations). The exact nature of the signaling complex is currently unclear.

Antibodies to the TNF-R55 (49, 50), Fas receptor (21, 35, 51), CD27 (52), and CD40 (53) have been shown to have receptor-activating properties. Presumably antibodies

Table 4. *Summary of the Effects of TNF, Anti-Fas, LT-α₁/β₂, and Anti–LT-β-R on the Growth of Various Cells in the Presence of IFN-γ*

Name	Type	Growth inhibition by			
		TNF	Anti–Fas	LT-α₁/β₂	Anti–LT-β-R
Brain					
U118	Glioblastoma	+/−	+/−	−	−
SW1783	Astrocytoma	−	++	−	−
SW1088	Astrocytoma	+	+/−	−	−
Skin					
A375	Melanoma	−	−	−	ND
SK-MEL-1	Melanoma	−	−	−	−
Colorectal					
HT29	Adenocarcinoma	++	++	++	++
WiDr	Adenocarcinoma	++	++	++	++
SK-Co-1	Adenocarcinoma	++	++	−	−
SW 403	Adenocarcinoma	−	++	−	−
SW 480	Adenocarcinoma	+	+	−	−
SW 620	Adenocarcinoma	−	−	*‡	−
SW 837	Adenocarcinoma	+/−	+/−	−	−
SW 1116	Adenocarcinoma	−	+	−	ND
SW 1417	Adenocarcinoma	−	−	‡	ND
Colo 320DM	Adenocarcinoma	−	ND	−	ND
LoVo	Adenocarcinoma	+	+/−	−	−
DLD-1	Adenocarcinoma	+	+	−	+/−
LS 174T	Adenocarcinoma	++	+/−	−	−
LS 123	Adenocarcinoma	+	−	−	ND
T84	Carcinoma	+	++	−	−
HCT 116	Carcinoma	+/−	−	−	ND
NCI H508	Adenocarcinoma	++	+	−	ND
CACO-2	Adenocarcinoma	−	−	−	−
Breast					
BT-20	Carcinoma	++	−	−	ND
SK-BR-3	Carcinoma	+/−	−	+/−‡§‖	−
MCF-7	Adenocarcinoma	++	+	+/−‡‖	ND
MDA-MB-468	Adenocarcinoma	++	ND	+/−§	+
Cervix					
ME180	Carcinoma	+	+	+/−‖	+
HT-3	Carcinoma	++	−	++‡	+/−
MS751	Carcinoma	+ª	+/−	−	ND
Ovary					
SK-OV-3	Adenocarcinoma	−	−	−	−
Pancreas					
PANC-1	Epitheloid carcinoma	+	+	−	−
Capan-1	Adenocarcinoma	−	++	−	−
Capan-2	Adenocarcinoma	−	++	−	−
Lung					
A549	Carcinoma	+/−	ND	−	ND
Lymphoid					
U937	Histiocytic	++	+/−	+/−‖	ND
K562	Promyelocytic	−	−	−	−

*Some growth stimulation.
‡Altered morphology.
§Heterogenous cell population, responders and nonresponders present.
‖Unconfirmed if LT-α component.

LT-β R ceptor **CD40**

HT-29
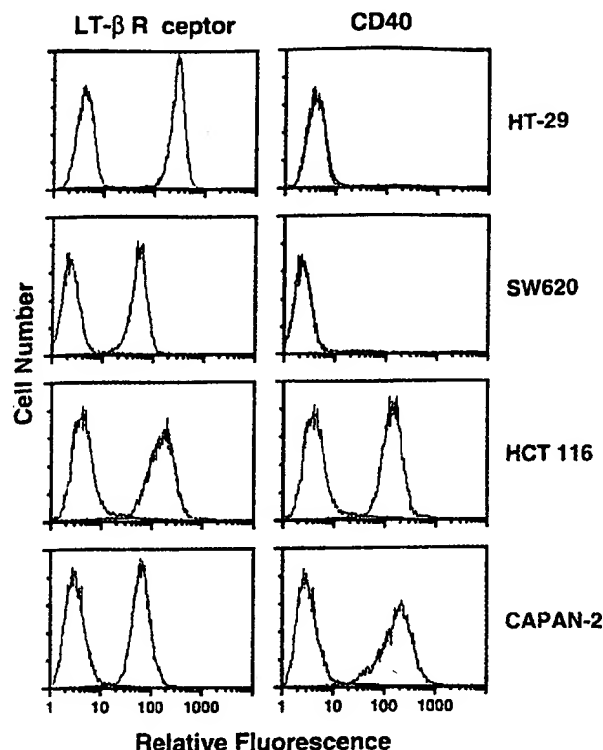
SW620

HCT 116

CAPAN-2

Cell Number

Relative Fluorescence

Figure 5. FACS® analysis comparing CD40 and LT-β-R levels on three colorectal carcinoma lines, HT-29, SW620, HCT 116, and the pancreatic carcinoma CAPAN-2. Untreated cells were stained with anti-CD40 (BB20), anti-LT-β-R (BDA8), or a control mAb (MOPC 21). Control antibody staining is the curve with the lowest staining in each panel.

A

B

Injections

Tumor Size cc

Days post inoculum

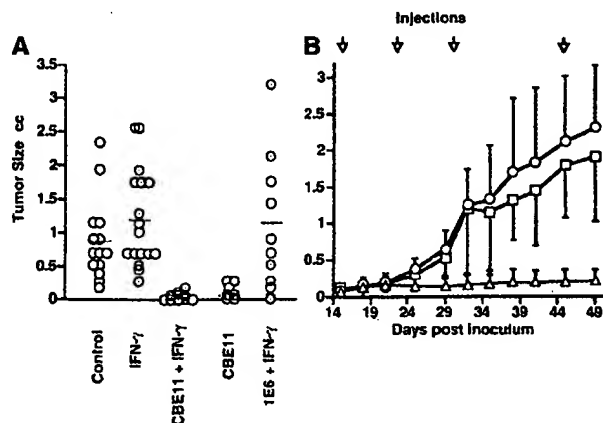Control   IFN-γ   CBE11 + IFN-γ   CBE11   1E6 + IFN-γ

Figure 6. Growth of the human adenocarcinoma WiDr tumor in SCID mice. A shows the size of tumors 30 d after inoculation. Mice were treated on days 0 and 1 with saline, IFN-γ alone, an anti–LT-β-R mAb CBE11 with and without IFN-γ, and a control anti–human LFA3 mAb 1E6 with IFN-γ. Animals treated with CBE11 or 1E6, represented by circles with dots, received 10 μg/mouse per injection of antibody instead of 50 μg for the open-circle animals. The mean of each group is indicated by a crossbar. Means, standard deviations, and (number of animals) for the five groups (left to right) were 0.88 ± 0.59 (14), 1.21 ± 0.7 (21), 0.041 ± 0.052 (16), 0.11 ± 0.1 (12) and 0.98 ± 1.16 (12). B shows the growth of WiDr tumors as per A, however, the tumors were grown to an average diameter of 0.53 cm (0.076 cc) without any treatment, then intraperitoneal injections were started on day 15 and continued as indicated by the arrows. Animals were treated with IFN-γ alone (10⁶ U/injection) (□), IFN-γ with 50 μg 1E6 anti-LFA-3 (○), or IFN-γ with 50 μg CBE11 anti-LT-β-R (△). Means and standard deviations are indicated for groups of 12 animals.

capable of cross-linking the receptors in the right orientation will generate receptor aggregates that can signal. Dimerization of the TNF-R55 may be sufficient to trigger TNF signaling; however, in the Fas receptor case, larger aggregates need to be formed (54). When immobilized on a plastic surface, all the anti–LT-β-R mAbs were able to induce death, including those that blocked ligand binding. Similar patterns were shown for anti-Fas receptor mAbs (51). In contrast, the anti–LT-β-R mAbs were not very effective in solution unless mAbs to two different epitopes were mixed together. In this case, it can be envisioned that aggregates larger than dimers could form, resulting in more productive signaling. When combined with the LT-α₁/β₂ ligand, some antibodies blocked activity, presumably by directly blocking the binding site, e.g., BDA8 and AGH1, although there was some evidence for mixed agonistic/antagonistic effects even in this case. At the other end of the spectrum, some mAbs, e.g., CDH10 and BKA11, potentiated the killing induced by ligand. Again, cross-linking of the receptor into small aggregates presumably facilitates the ability of ligand to productively cross-link and signal. Interestingly, the synergistic effect of the anti–LT-β-R mAbs with the LT-α₁/β₂ ligand also occurred between anti-LT-β-R mAbs and TNF. We do not have any evidence that the TNF receptors are involved in LT-β-R signaling, and

therefore we interpret the effects on TNF signaling as resulting from more complex priming-like events at the intracellular level. Anti–LT-β-R mAbs can trigger NF-κB activation without inducing cell death (MacKay, F., manuscript submitted for publication), thus it is possible that both the LT-β-R and TNF-R intracellular signal transduction pathways utilize some common elements resulting in synergistic cross talk. Along with the soluble ligands and LT-β-R-Fc forms, the anti–LT-β-R mAbs are good tools for either activating or inhibiting the LT-β-R signaling pathway.

TNF can induce necrosis, apoptosis, or mixed mechanisms, depending on the cell line (55, 56), whereas Fas-triggered death is generally described as occurring by apoptosis. TNF can have either fast or slow effects on cells, probably reflecting multiple mechanisms (57). The experiments described here showed a lack of classical DNA laddering, although some DNA cleavage was detected using TUNEL techniques. The nuclei appear to condense, yet the cytoplasm balloons out in a manner characteristic of necrosis. It is possible that the ballooning occurs long after the death event and is observed in these assays, which are relatively long term compared with conventionally studied apoptotic events. Morphologically, the death induced by TNF, anti-Fas receptor, and LT-α₁/β₂ are similar, differing only kinetically. The signaling pathways initiated in each case may be different, as shown previously for Fas and TNF receptors (58, 59). The action of TNF is often accelerated by

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the addition of cycloheximide to the culture, and the HT-29 cells are no exception. In contrast, LT-α_1/β_2 lacked activity in the standard short term cycloheximide-containing assay. Either the LT-α_1/β_2 action required protein synthesis, or the cytotoxic events are simply too slow to manifest themselves in the short-term assay format. In the TNF case, the signaling pathways leading to death are likely to be different depending on whether or not cycloheximide is present (60). The effects of cycloheximide and the slow pace of LT-β-R–induced death relative to TNF and Fas suggest that LT-β-R acts via a different pathway. This concept is intriguing since the LT-β-R lacks a canonical death domain. Either receptor cytoplasmic domains lacking the death domain can signal death, or the LT-β-R undergoes complex ill-defined interactions with other receptors. In light of the recent observations on the slow death mediated by TNF-R75 (23, 24), which also lacks a canonical death domain, it is possible that there are other death pathways used by some TNF family members distinct from that typified by the well-studied Fas system.

The ability to induce death selectively in tumor cells is an attractive goal. The signaling mediated by the TNF family of receptors is intriguing since this is one of relatively few cases in which a normal physiological signal can induce cell death as opposed to the loss of a signal, e.g., IL-2 or IL-3 removal. The use of TNF to treat cancers was predicated on this concept even before the emergence of programmed cell death as an important physiological process. Activation of the LT-β-R with the CBE11 mAb in vivo blocked the growth of WiDr cells inoculated into SCID mice. Since the anti-human LT-β-R mAb cannot bind to mouse cells, the growth inhibition mechanism must directly target the tumor cell. Likewise, the exogenously added IFN-γ can act only on the tumor cells since mouse IFN-γ does not bind to human IFN-γ receptors (61) and human IFN-γ does not bind to mouse IFN-γ receptors. Therefore, because of the lack of an absolute requirement for IFN-γ, it remains unclear whether the in vivo effect reflects direct cytotoxicity as observed in vitro or whether other mechanisms are contributing. If other mechanisms are involved, a wider range of tumors may be affected in vivo than would be forecast by the in vitro analyses. Generally, more primary tumors were found to be affected by TNF in in vivo models than would be surmised from the analysis of in vitro cultured tumor lines (62).

An LT-β-R–based antitumor strategy may be important when considered in the context of recent advances. TNF, Fas, and LT-α_1/β_2 are clearly cytotoxic to tumor types with mutant p53 such as HT-29 (63), and, moreover, the TNF signaling pathway in cells of fibroblastoid or epithelial origin is not sensitive to the protective effects of bcl-2 (64). Therefore, the induction of a death sequence might occur via a route that circumvents the wild-type p53 dependency of some chemotherapeutic approaches (65). A proper understanding of these signaling processes may lead to alternative strategies for controlling cancer. Clinically, isolated limb perfusion with TNF dramatically demonstrated the soundness of the approach (66), but unfortunately the systemic application of TNF was frustrated by dose-limiting toxicity resulting from activation of inflammatory cascades. Likewise, the Fas receptor is widely distributed, and anti-Fas mAbs can be very potent inducers of apoptosis; however, receptor activation also leads to rapid necrosis of normal liver cells and death in mice. This activity would certainly complicate its therapeutic application (67). The LT-β-R is present on most transformed cell types, and its activation also presents a potential anticancer therapy. Moreover, it is likely that sufficient IFN-γ exists in the tumor environment to synergize with LT-α_1/β_2 without exogenous administration (68). Our preliminary data indicate that LT-α_1/β_2 does not effectively activate primary human endothelial cells to express the VCAM or E-selectin adhesion molecules, and hamster anti-m-LT-β-R mAbs do not cause death (Hochman, P., G. Majeau, F. Mackay, and S. Browning, manuscript submitted for publication); therefore, LT-β-R signaling should lack TNF-type toxicity. If the physiological activity of LT-α_1/β_2 is limited to more subtle regulatory effects on lymph node function, it is conceivable that an LT-α_1/β_2 therapy may provide an alternative therapy for some adenocarcinomas.

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